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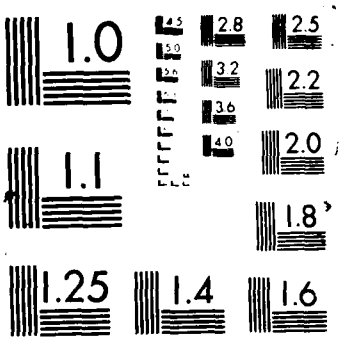
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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

In the first year of this project we have isolated and cloned two rRNA operons of Halobacterium marismortui, each coding for 5S, 16S and 23S rRNA genes. The structures of the two operons were compared by restriction mapping, by hybridization of different restriction fragments to ^{32}P labelled rRNA molecules and by R-loop analysis. The two operons are not identical. The direction of transcription was determined to be 5'-16S-23S-5S-3' and tRNA genes were also identified. The promoter regions of the two operons as well as their 16S rRNA maturation sites were determined using S1 analysis. One operon has one promoter 270 bp upstream from the matured 16S rRNA and the other operon has three promoters in tandem at distances of 295-470bp upstream from the matured 16S rRNA. Sequencing of these regions is currently underway. Protocols are being developed to perform footprint analysis at high salt concentration, for the characterization of the polymerase binding site in the promoter region. A major aim is to reconstitute a promoter dependent transcriptional assay.

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Title: Study of Pure Proteins, Nucleic Acids and their Complexes from Extreme Halobacteria of the Dead Sea: RNA Polymerase-DNA Interaction.

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Table of Contents

1. Project Goals

The objectives of our program are to isolate and characterize a fully active DNA dependent RNA polymerase from the extremely halophilic archaebacteria of the genus Halobacterium; to isolate and characterize promoter regions of these bacteria; to study the specific polymerase-DNA interactions.

Our goals for the first year were to isolate the promoter regions of the two rRNA genes of H. marismortui and to use these genes for the development of a specific transcriptional assay for the RNA polymerase. Since it is not clear what are the control elements for transcription the rationale for choosing these operons was to choose genes which are heavily transcribed.

One of the problems in isolating fully functional RNA polymerase is the development of a good enzymatic assay. Although attempts to purify the enzyme from H. halobium were partially successful, the purified enzyme could not initiate transcription specifically (F. Gropp and W. Zillig, personal communication). A possible reason for the lack of specific initiation is the loss of initiation factors during the purification of the enzyme. It was, thus, essential that we should be able to detect either transcription that initiates specifically at a promoter site or binding of specific factors or enzyme complexes to the promoter region. Once specific transcription or promoter binding could be demonstrated we could use these as an assay for purification of these proteins.

2. Accomplishments

A) Isolation and characterization of two rRNA operons in H. Marismortui.

The halobacteriaceae are extreme halophiles. They require 3.5 M NaCl for optimal growth and no growth is observed below 2 M NaCl (1). Their intracellular salt concentration reaches 4.5 M KCl and 1.4 M NaCl (2); thus, all their biochemical machinery is adapted to function at high salt concentrations. Although various biochemical aspect of Halobacteria have been studied (3) very little is known about the organization of their genome. Though the genes for bacteriorhodopsin (4) and rRNA (5) were isolated from the genome of H. halobium a detailed genetic study was difficult to perform due to the extreme genetic instability in this strain (6). In contrast, the genome of the extreme halophilic and prototrophic bacterium H. marismortui, isolated from the Dead Sea (7), is extremely stable. Hence we present data concerning the rRNA operons of H. marismortui.

Total bacterial DNA was digested with Hind III restriction endonuclease and the fragments were fractionated on sucrose gradients. The DNA fractions were transferred to nitrocellulose filters and hybridized to a mixture of 16S and 23S ^{32}P-labelled rRNA. The RNA hybridized strongly to two fractions which contained DNA fragments of 20 kbp and 10 kbp respectively. These fragments were cloned into E. coli HB101, isolated and shown to hybridize to purified 16S as well as to purified 23S rRNA, indicating the existence of two operons of the rRNA genes. The

smaller 10 kbp Hind III fragment was designated HH10 and from the larger 20 kbp fragment an 8 kbp Hind III - Cla I fragment (designated HC8) containing the entire rRNA operon was isolated. Subcloning into pBR322 yielded the plasmids pHH10 and pHC8.

Plasmids pHH10 and pHC8 were digested with ten different restriction enzymes. BamH1 and Bgl II did not cleave the cloned DNA. The restriction maps of the other eight enzymes were established by a series of digestions and double digestions of the plasmids with different combinations of these enzymes. The resulting maps are given in Figure 1. The positions of the genes for the different rRNA species were determined by (a) digestion with several enzymes, blotting and separate hybridization to 16S, 23S, 5S rRNAs and to tRNA (Figure 2) and (b) heteroduplex formation between 16S+23S rRNA and the cloned DNA, and electronmicroscopy analysis (Figure 3). (c) S1 analysis of the 5' and 3' ends of the mature 16S and 23S rRNA. Using the latter technique the size of the 16S rDNA was estimated to be 1450 bp, in agreement with the size of 16S rRNA of H. volcanii (1472 residues) (8), and the size of 23S rDNA was estimated to be 2800 bp.

The structures of the two operons differ in several aspects: (a) an extra Xho I site exists in the beginning of the 23S rDNA of HH10. (b) The spacer region between the 16S and the 23S rDNA is 600 bp long in HH10 and 800 bp long in HC8. Restriction enzyme polymorphism in rRNA genes is known in eubacterial systems. In E. coli for instance an EcoR1 site exists in the 23S rRNA coding region in 6 rRNA operons and is missing in rrnF, and a Hind III site exists in the 16S rRNA coding only in the rrnB operon (9).

Restriction fragments that contain the spacer region or the region of the 5S rDNA hybridize to tRNA. The existence of tRNA genes in these regions was documented in E. coli (10) and in the archaebacterium Methanococcus vanielii (11). It is thus possible that in H. marismortui there are tRNA genes in these regions.

To determine the direction of transcription the 1.8 kbp EcoR1 fragment of pHC8 was subcloned into the ssDNA bacteriophage M13 mp8. Plaques that hybridized to ^{32}P labeled 23S rRNA, and therefore contained the complementary strand, were selected. The orientation of the cloned fragment was determined by digestion of the RF of the recombinant bacteriophage DNA with PstI and EcoR1 + EcoRV restriction endonucleases. Since the PstI site in the recombinant phage DNA is at the 3' side of the EcoRV it should be at the 5' side of it in the complementary strand, which has the orientation of the rRNA. Taking into account the restriction map of pHC8 (Figure 1) and assuming that all the rRNA molecules are transcribed in the same direction it can be concluded that the direction of transcription is 5'-16S-23S-5S-3'.

B) S1 analysis of rRNA operons

The specific goals were

(i) to define the boundaries of the 16S and the 23S rRNA genes within the two cloned restriction fragments of genomic DNA.
(ii) to determine if the two gene sets are identical or nearly identical.
(iii) to demonstrate that both operons are actively transcribed during growth of H. marismortui
(iv) to characterize similarities or differences that might exist in the control regions driving the expression of the two separate operons.

Using 5' or 3' end labelled restriction fragments (labelled at conserved positions) from the two clones in S1 nuclease protection experiments with RNA isolated from growing cells, the 5' and 3' boundaries of the respective 16S and 23S gene have been defined. The two 16S and 23S gene copies are virtually identical in length and sequence although some heterogeneity, demonstrated by restriction site polymorphism, is apparent. The 5' flanking and the 16S-23S intergenic regions in the two operons are quite different. The HC8 operon contains a 600 bp intergenic space and apparently contains three tandem transcription start sites located about 470, 370 and 295 bp in front of the 16S gene. The HH10 operon in contrast contains a shorter 450 bp intergenic space and contains only a single promoter located at about 270 bp in front of the 16S gene. Both operons are apparently transcribed; this conclusion is based upon the fact that multiple 3' and 5' transcript ends located within the 16S-23S intergenic space originating from alternate processing of the two primary transcripts have been visualized. The results of the S1 analysis are included in Figure 1.

C) Promoter sites sequencing

Because of the clearly different 5' regulatory region for the two operons, DNA sequence analysis has been initiated. About 1500 nucleotides of sequence have been determined from the 5' flanking region and within the 16S gene of the HC8 operon. The sequence analysis is in progress.

D) Footprint analysis of rRNA polymerase promoter interaction

Halophilic archaebacteria have adapted to environments that are nearly saturated in salt in two ways; first by maintaining an equilibrium between the intra- and extracellular ionic strength and second by evolving enzyme systems with novel properties which allow them to function in these high ionic strength conditions. Clearly under these conditions, ionic interactions between macromolecules will be greatly diminished. We have, in the past, been concerned with the definition of biophysical characteristics of protein molecules which allow them to function in high salt environments and to understand the nature of protein-DNA and protein-protein interactions in high salt (11,12). The regulation of gene expression is mediated through

the binding of proteins and effector proteins to specific promoter sequences within genomic DNA. The binding of DNA dependent RNA polymerase and associated effector proteins to specific promoter sequences obtained from genomic DNA serves as a model for studying these interactions. Restriction fragments containing the transcription start sites are being subcloned and will be utilized in binding assays to identify proteins from crude cell extracts which bind to these regulatory sequences. When binding is detected, the proteins can be purified and the biophysical nature of the DNA-protein interaction can be studied.

At the high salt concentrations in the extreme halophilic systems the standard methods of filter binding and DNAse I footprint analysis run into difficulties. We are therefore working on adapting to high salt conditions a new approach, recently introduced (13-15), in which footprint analysis of protein nucleic acid complexes is performed by chemically generating hydroxyl radicals in solution to cut uncomplexed DNA backbones.

E) Attempts at performance of in vitro transcription assay.

As a first step in the isolation of fully functional RNA polymerase we tried to develop a functional assay. Our first attempt was to transcribe the cloned rRNA operon and analyze the products. Unfortunately we could not detect any specific transcription when crude extract of H. marismortui was used as the polymerase source. In a control experiment a specific transcription of E.coli genes (located on the pBR322 cloning plasmid) could be detected using E.coli polymerase. These experiments will be taken up again when polymerase components and effectors will become available following the footprint analysis.

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G) Legends for Figures

Figure 1: Restriction map of the HC8 and HH10 operons of the rRNA genes of H. marismortui. The darkened area indicate the coding regions. The enzymes used for the map are: C-Cla I; E-Eco RI; H-Hind III; K-Kpn I; P-Pst I; S-Sal I; Sm-Sma I; V-Eco RV; X-Xho I. a few sites of the enzyme Ava I are indicated by A. The lower arrows indicate processing sites and the upper arrows indicate the putative promoter sites.

Figure 2: Agarose gel electrophoresis of restriction fragments of pHH10 (A) and pHC8 (B) (lane a) and the corresponding autoradiographs of Southern hybridization to ^{32}P labelled 16S (lane b) and 23S (lane c) rRNA. Fragments that appear weakly in the autoradiograms are indicated by arrows.

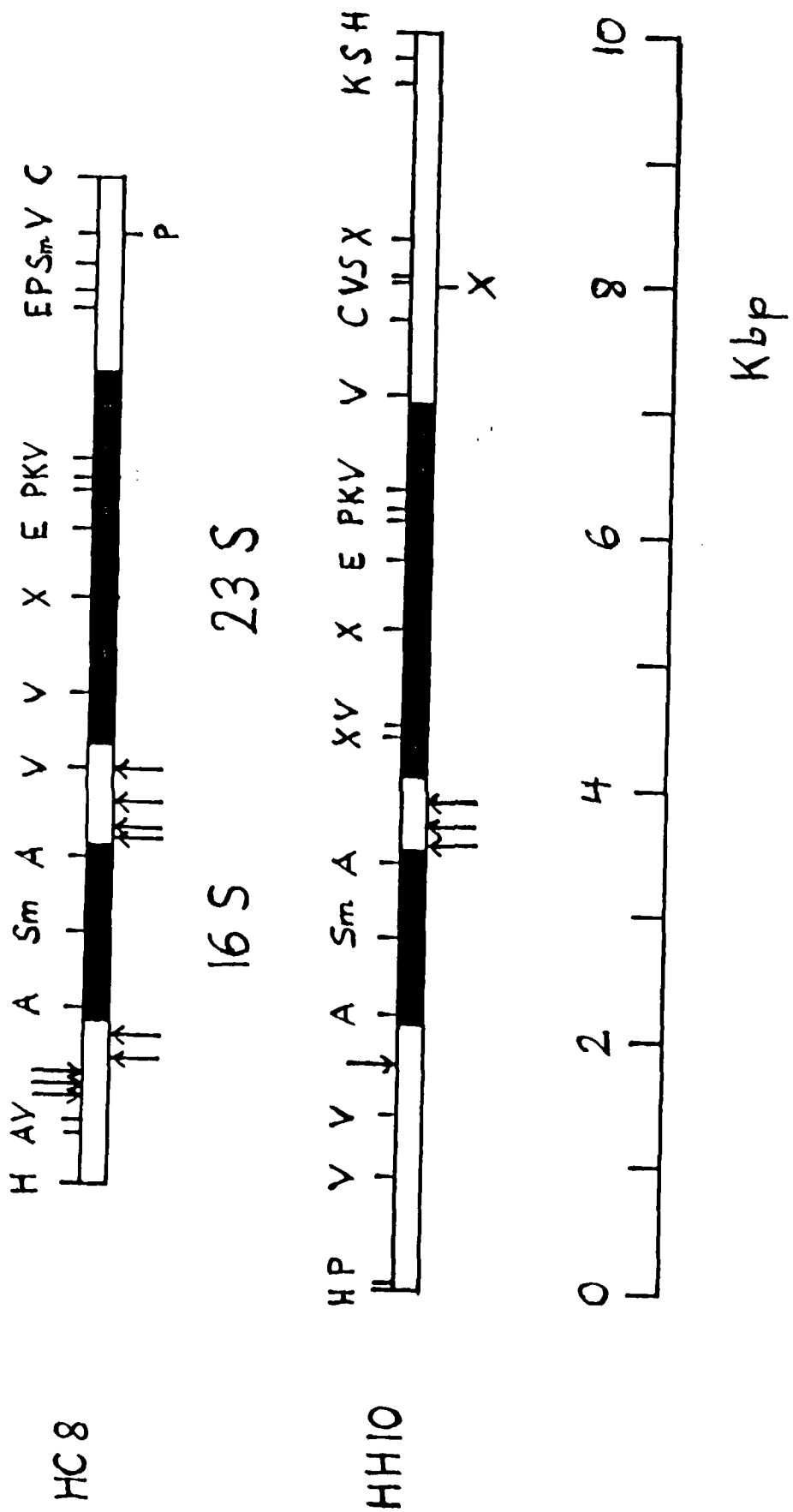
Figure 3: Electron micrographs of R-loops formed between H. marismortui 16S and 23S RNA and the 10kbp Hind III fragment of pHH10 (A) and the 8kbp Hind III-Cla I fragment of pHC8 (B).

3. Conclusions and plans.

Results presented in this report are now being prepared for publication. Work in the coming year will continue along the same lines as discussed in this report. Sequencing of the promoter regions of the two rRNA operons HH10 and HC8 will be completed. We would like to isolate additional genes and determine their promoters sites to be able to define a consensus sequence of halobacterial promoters, if there is such a consensus sequence. In parallel footprint analysis will be used to determine the nucleotide sequence of the polymerase binding site in the promoter region. The specific binding assay can then be used as a probe in the purification of the polymerase or its transcriptional factors. Once these factors will be purified they will be used to attempt reconstitution of a promoter dependent transcriptional assay.

4. Personnel.

This grant was activated on April 1, 1986. We then requested and were granted a six months extension without additional funds. Actual work was started on October 1, 1986 and the due date for the first annual progress report is October 1, 1987. Yohevet Lamed, M.Sc. joined the program on October 9, 1986 and Emanuel Yakobson, Ph.D. on December 7, 1986. On May 1, 1987, Sarah Lefkovitz, Ph.D., a postdoctoral student, joined the group. We have initiated a collaboration with Professor Patrick P. Dennis, of the University of British Columbia, Vancouver, Erna and Jacob Michael Visiting Professor at the Weizmann Institute in our laboratory, during his stay here (May to August 1987).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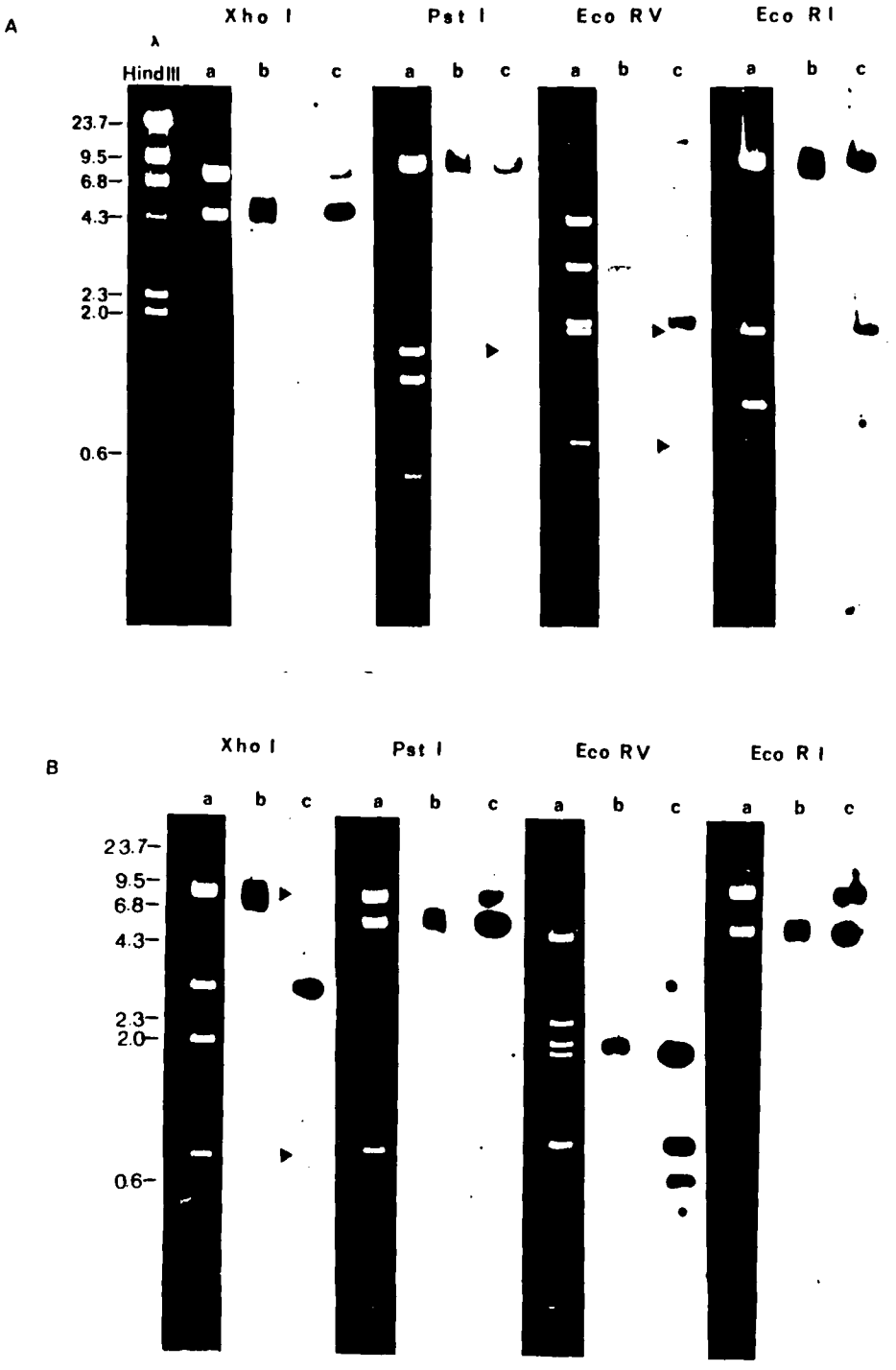
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Figure 2

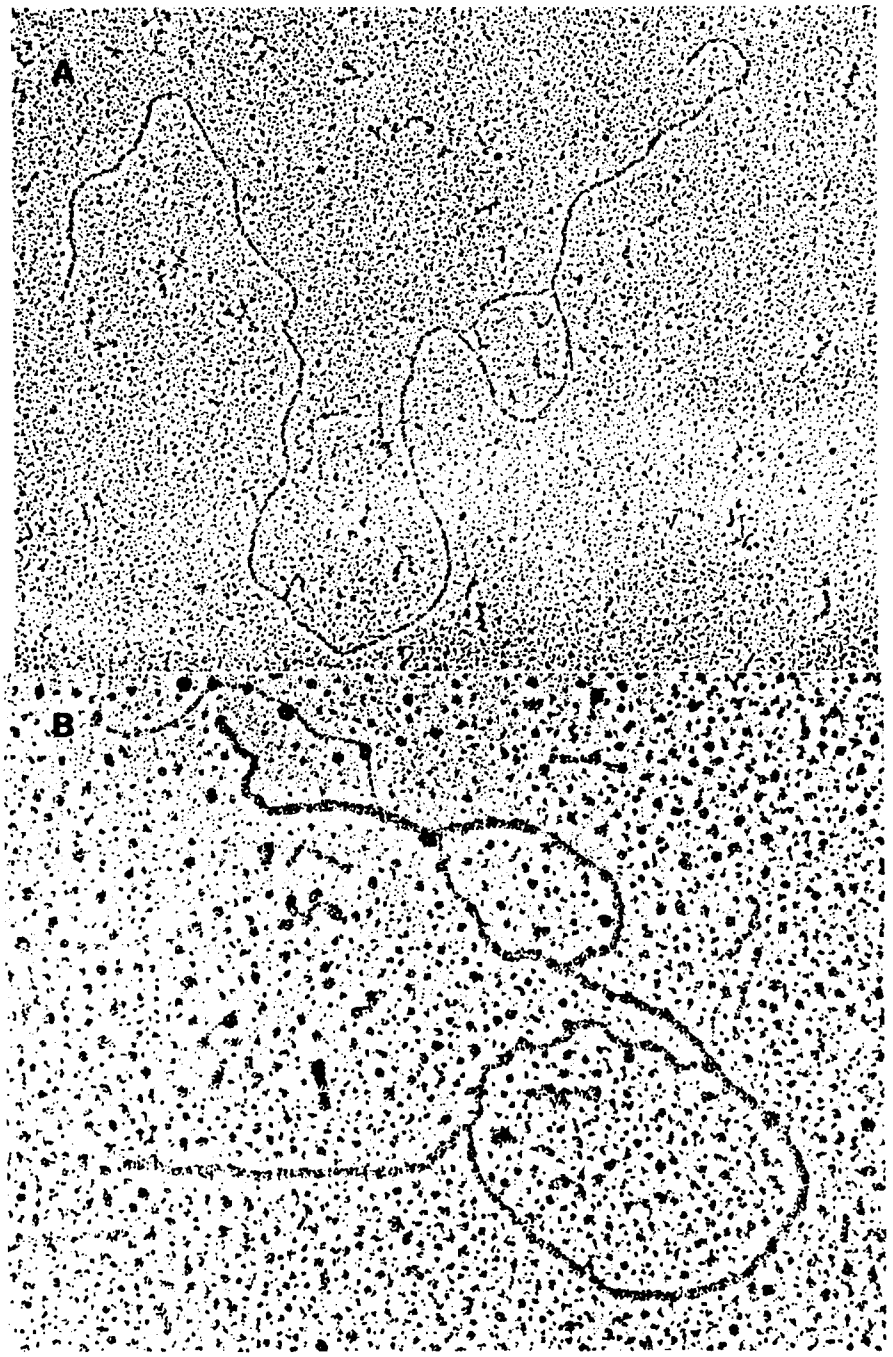

Figure 3

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